CHAPTER-4

Fundamentals with pictures

(A picture says more than a thousand words)

4.1 Chromatography

In chromatography, a physical separation method, the components of a mixture are partitioned between two phases. One of the phases stays in its place and is called the stationary phase, whereas the other moves in a definite direction and is called the mobile phase.

According to the type of mobile phase we distinguish between gas chromatography, supercritical fluid chromatography, and liquid chromatography. The separation is based upon the different partition coefficients of the sample components between the two phases. It is helpful to divide the chromatographic column into small hypothetical units, the socalled theoretical plates. Within each plate a new partition equilibrium is established. The narrower a theoretical plate, the more equilibrium processes can take place within a column of given length and the more demanding the separation problems which can be solved.

The figure-1 shows the separation of two compounds. One of these prefers the mobile phase but also enters the stationary phase. For the other compound the preference is the other way round. Thanks to this large difference in their properties the two types of molecule can easily be separated. They are transported through the column by the flow of the mobile phase and thereby reach zones where new equilibria are formed again and again

In the drawing, such a theoretical plate has a height of approximately 3 1/2 stationary phase particle diameters. This height depends on the packing quality of the column, on the mass transfer properties of the phases, and on the sample compounds involved. Plate height is a function of the particle diameter of the stationary phase. For good columns, plate heights are equal to ca. 3 particle diameters irrespective of the particle size. A fine packing, e.g. with a 5 μm phase, gives four times as many theoretical plates as does a 20 μm packing if identical column lengths are compared. The column with the fine packing can therefore be used for more difficult separation problems. [165]

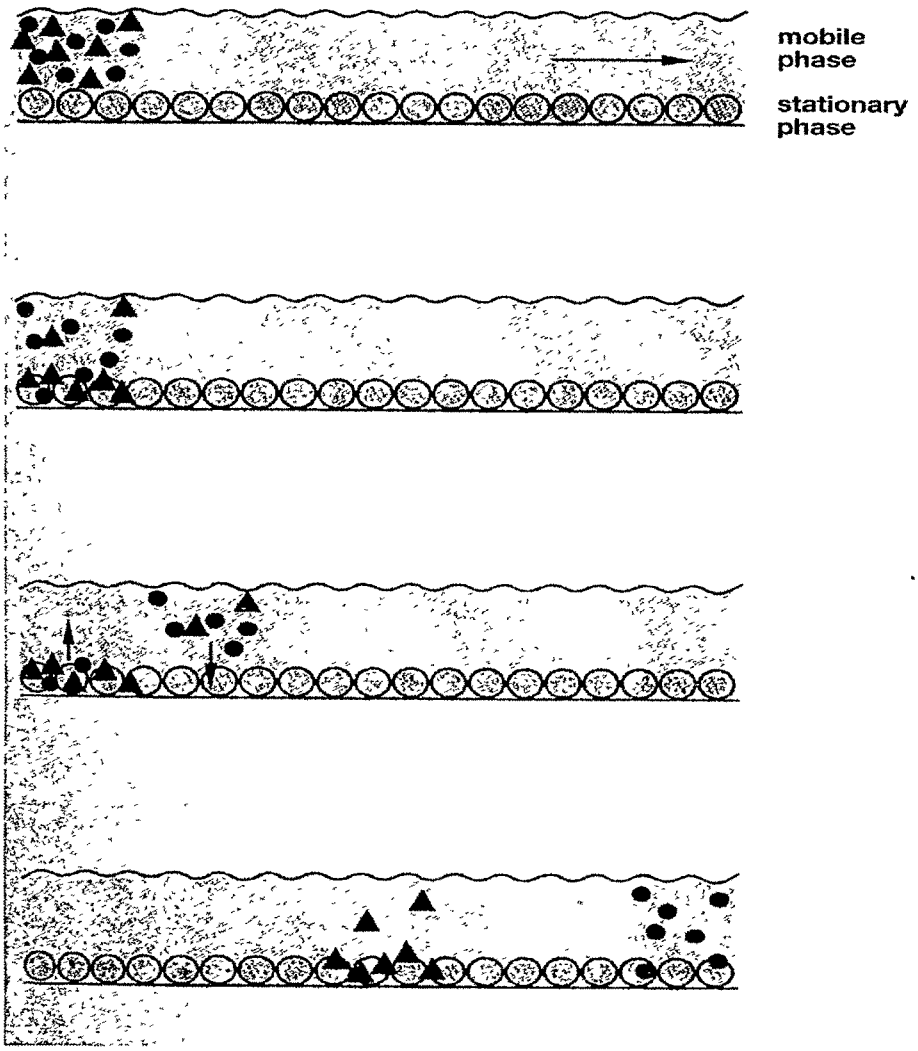


Figure-1

4.2 Chromatographic Figures of Merit

To judge a chromatogram it is necessary to calculate some data which can be easily obtained. The integrator or data system yields the retention times, *tR,* and peak widths, w; perhaps it is advisable to determine the peak width at half height, $w_{1/2}$. In addition the breakthrough time or 'dead time', *to,* must be known although it can be a problem to measure it unambiguously. In principle, the first baseline deviation after injection marks *to.* Then the following data can be calculated·

1. Retention factor, κ (formerly capacity factor, κ'):

$$\kappa = t_R - t_o / t_o$$

The retention factor is a measure of the retention of a peak. It depends only on the phase system (the types of mobile and stationary phase) and on the temperature.

2. Separation factor, α

$$\alpha = \kappa_2 / \kappa_1$$

Two compounds can be separated only if α is higher than 1.0 in the selected phase system. For HPLC separations α should be 1.05 or higher

3. Theroetical plate number, N.

$$N = 16(t_R / w)^2 = 5.54(t_R / w_{1/2})^2 = 2\pi\ (hpt_R / A_p)^2$$

where h_p = peak height and A_p = peak area. The plate number is a measure of the separation performance of a column. (The equations given here are in principle only valid for symmetrical peaks)

From the plate number it is possible to calculate the height, H, of a theoretical plate (e.g., in μm):

$$H = L_c / N$$

where L_c = column length

4. Tailing T (for asymmetric peaks)

$$T = b/a$$

where a and b are determined at 10 % of peak height [165]

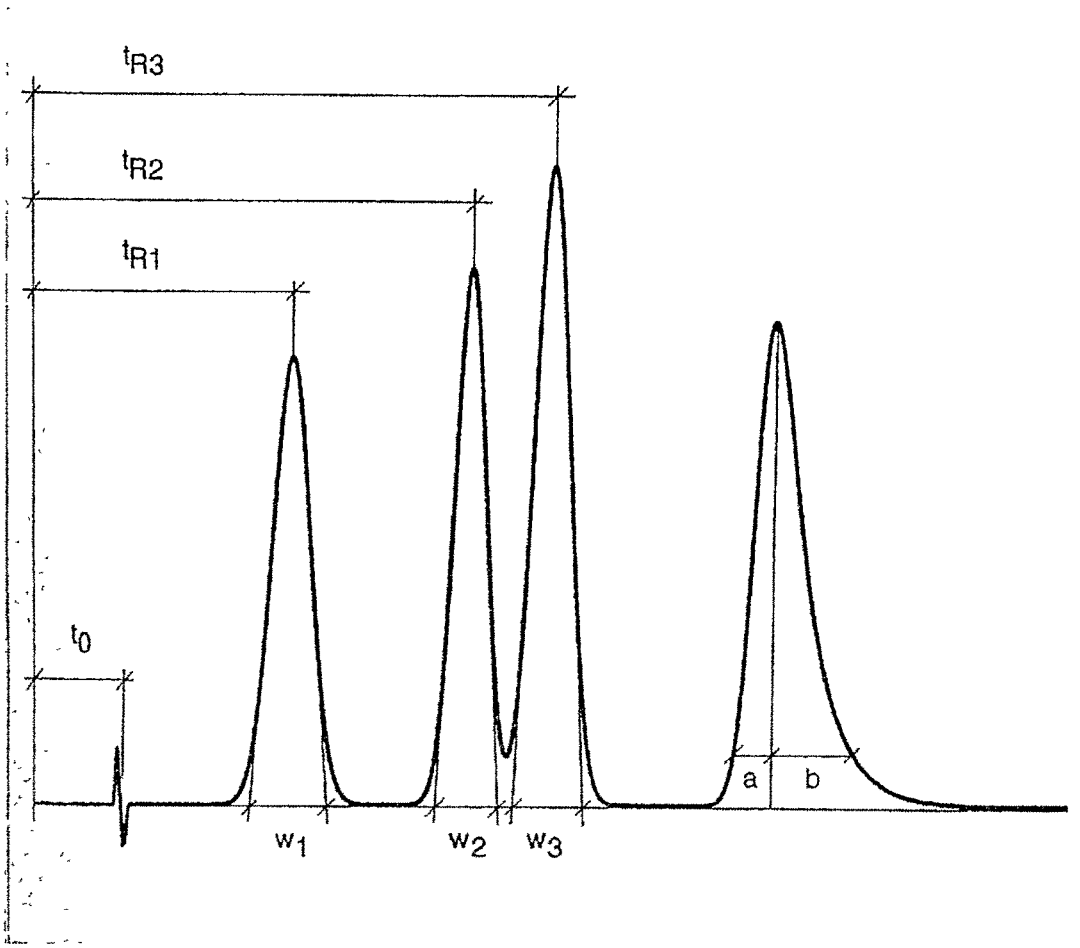


Figure-2

4.3 The Resolution of Two Peaks

The resolution of two adjacent peaks is defined as

$$R = 2\,(t_{R2} - t_{R1}) / W_1 + W_2 \quad = 1.18\,(t_{R2} - t_{R1}) / W_{1/2}1 + W_{1/2}\,2$$

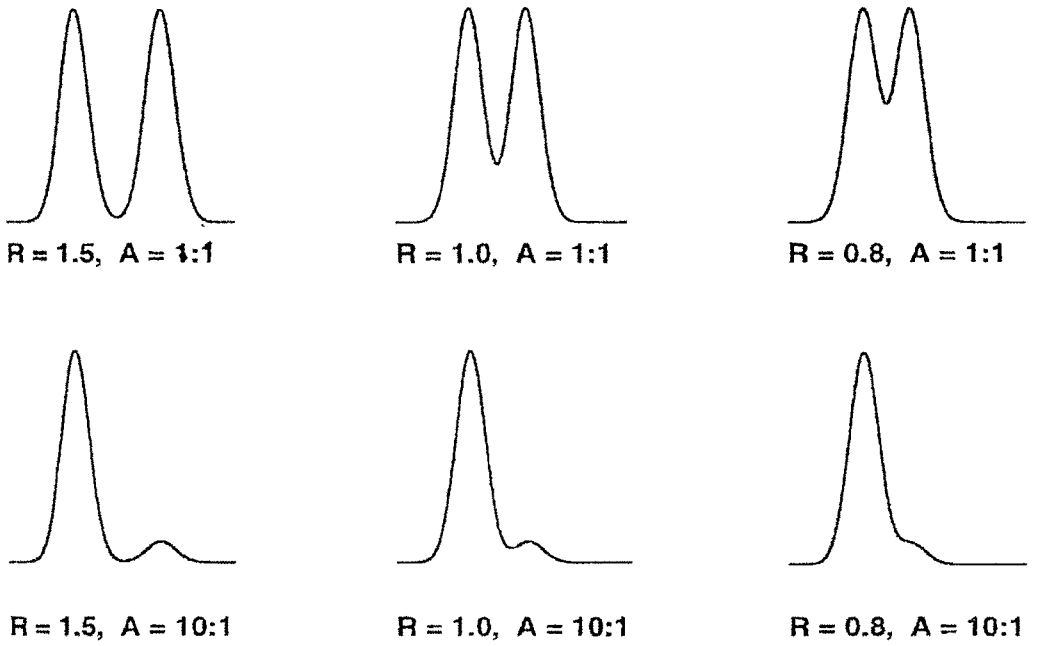
At a resolution of 1.0 the baseline between the peaks is not reached! Complete resolution is only obtained at R = 1.5 or higher, depending on the height ratio of the peaks. The smaller a peak compared with its large neighbor the greater is the resolution necessary to separate them.

The resolution depends on the separation factor α, the theoretical plate number N, and the retention factor k·

$$R = ¼ (\alpha-1) \sqrt{N} (k/ 1=k)$$

this equation can be expressed in different forms, which are not of interest here. It is important to realize that the resolution is influenced by the three parameters. The separation factor has the largest effect. If a separation needs to be improved it is well worth the effort of increasing α, although it is impossible to give a general proposal concerning how to do this. If the plate number is increased, the effect is only by the factor $\sqrt{N}$; if the column length is, e.g., doubled, and by this also the plate number (at least in principle), the resolution will improve only by $\sqrt{2} = 1.4$. Increasing the retention factor only has a notable influence on resolution if *k* was small to start with.

The upper figure presents several pairs of peaks separated with varying resolution The graph below demonstrates how the resolution increases with increasing plate number for three different separation factors [165]


R = 1.5, A = 1:1
R = 1.0, A = 1:1
R = 0.8, A = 1:1
R = 1.5, A = 10:1
R = 1.0, A = 10:1
R = 0.8, A = 10:1

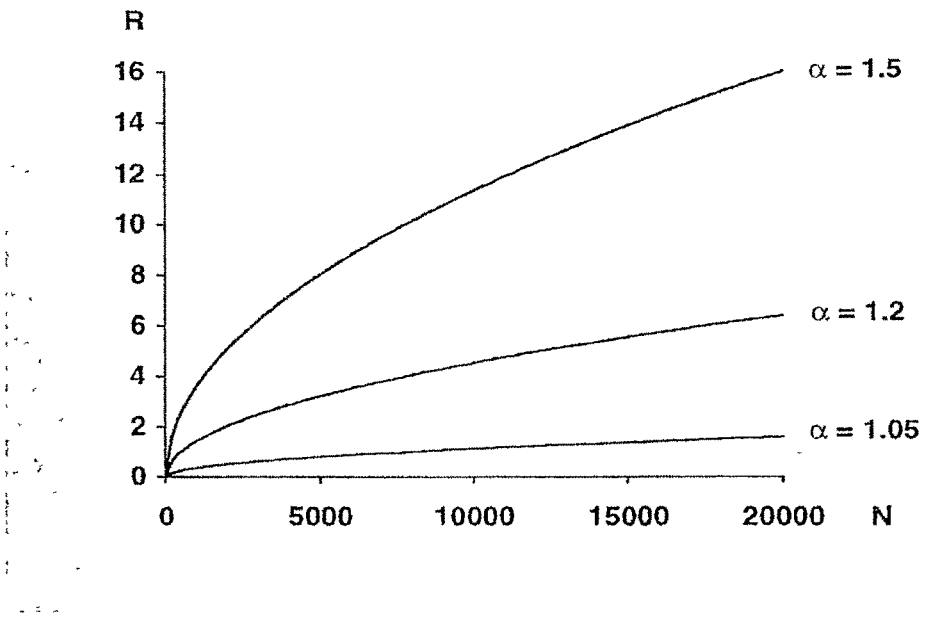

R
16
14
12
10
8
6
4
2
0
0
5000
10000
15000
20000
N
α = 1.5
α = 1.2
α = 1.05

Figure-3

4.4 Reduced Parameters

The judgement and comparison of HPLC columns is best done with reduced, dimensionless parameters. A test chromatogram is acquired which enables the theoretical plate number, N, of the column to be determined from a suitable peak with low tailing. It is also necessary to measure the breakthrough time, *to,* with a refraction index peak or with an otherwise suitable compound (for reversed-phase separations, e.g., with thiourea). The pressure drop, Δp, under the given conditions is noted.

Then the following reduced parameters can be calculated:

1. Reduced plate height, *h:*

$$h = H / d_p = Lc / N.d_p$$

h is a measure of the height of a theoretical plate as a multiple of the particle diameter, d_p. L_c is column length.

2. Reduced flow velocity, v:

$$v = u \,.\, d_p / D_m \, L_c \,.\, dp = L_c \, d_p / t_o \,.\, D_m$$

v is a measure of the flow velocity in relation to the particle diameter, d_p, and the diffusion coefficient, D_m. In most cases D_m is not really known but it can be assumed to be $1 \,.\, 10^{-9}$ $m^2 S^{-1}$ for small molecules in water/acetonitrile and to 4.10^{-9} $m^2 S^{-1}$ for small molecules in hexane.

3. Reduced flow resistance, *φ:*

$$\varphi = \Delta p \,.d^2_p / L_c . \eta \,.u = \Delta p \,.d^2_p . t_o / L^2_c . \eta$$

With φ the pressure drop can be described simply and clearly. It is, however, necessary to know the viscosity, η ,of the mobile phase. Mixtures of water and organic solvents pass through a maximum of viscosity!

Favorable numbers.

h=3

v=3

φ = 500 up to a maximum of 1000 [165]

4.5 The Van Deemter Curve

The separation performance of a column is not independent of the mobile phase flow rate. An optimum velocity, u_{opt} is observed where the performance is highest. This relationship is described by the van Deemter curve which describes the height of a theoretical plate, H, as a function of the linear flow velocity, *u*. At u_{opt} the plate height, H_{min}, is smallest, which means that the number of theoretical plates, N = L/H, is largest. The peaks are narrowest and thus eluted with the largest possible height; the resolution reaches a maximum. Any deviation from the van Deemter optimum yields smaller peak heights and resolutions; yet the optimum velocity is not identical for all compounds of a sample mixture.

It would be best to work at u_{opt}. Practical separations are often performed at higher speed, which gives shorter analysis times and usually only a moderate loss of separation performance. This is, however, only true if mass transfer is fast, which is often not the case with special stationary phases and ion exchangers. Of course it is never advisable to work under conditions left of the van Deemter optimum In this region the separation performance is very poor and the analysis time is long.

If the van Deemter curve is plotted with reduced parameters v and *h* the optimum is often at v = 3

Chromatographic conditions:

Sample : 5 µL of a solution of thiourea, veratrole, acetophenone and nitrobenzene

Column: 4.6 mm x 7.5 cm

Stationary phase: Zorbax SB C-18, 3.5 µ (reversed phase C18)

Mobile phase: water / ethanol 3 : 7

Flow rate: 0.1 to 1.9 mL min^{-1} corresponding to *u* from 0.16 to 3 2 mm S^{-1} Detector : 268 nm

Optimum : for nitrobenzene (curve) *u* ~ 1.3 mm S^{-1} (0.8mL min^{-1}), H ~ 8.5 µm; for veratrole and acetophenone *u* ~ 0.65 mm S^{-1} (0.4 mL min^{-1}) [165]

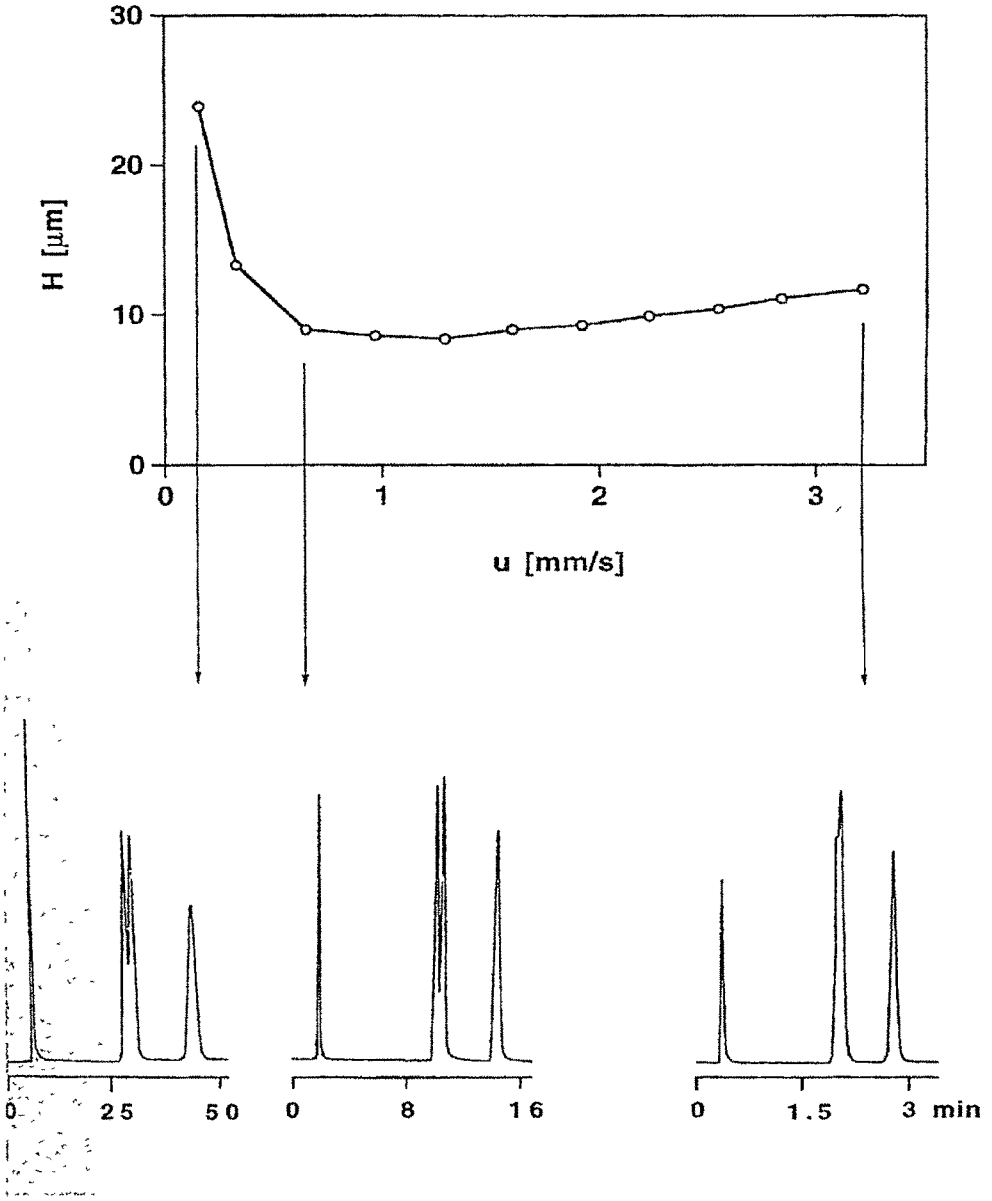


Figure-4

4.6 Peak Capacity and Number of Possible Peaks

For the separation of complex mixtures it is necessary to have space for as many peaks as possible with adequate resolution throughout the chromatogram. This number is the larger the higher the number of theoretical plates of the column and the longer one is willing to wait for the last peak. In other words, N and *k* determine the so-called peak capacity, n, of the column in use. Usually R = 1.0 is taken as the necessary resolution between two adjacent peaks .

If the plate number were constant over the whole range of *k* values the peak capacity would be defined as:

$$n = 1 + \sqrt{N}/4.\mathrm{Ln}\,(1 + k_{max})$$

At constant peak width, w, as could be the case with a very steep

solvent gradient, the peak capacity would be much higher:

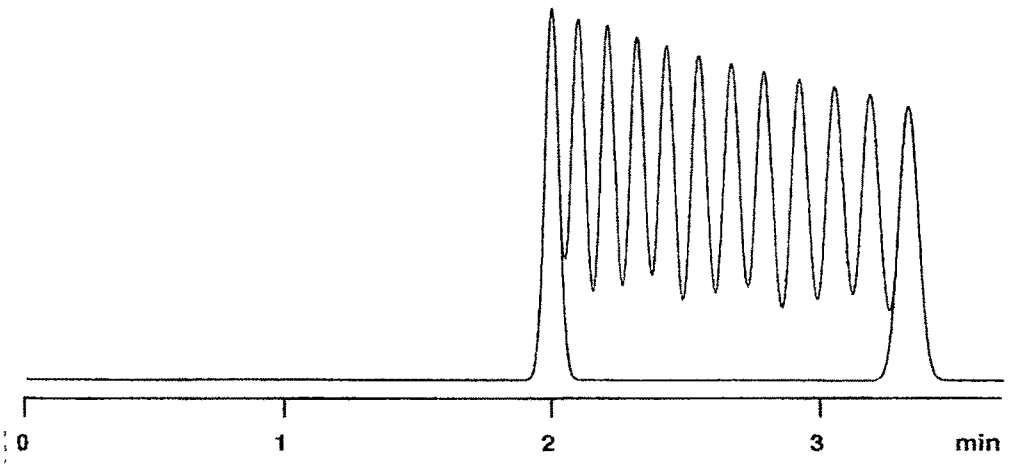
$$n = (t_R max - to\,) / W$$

In practice neither plate numbers nor peak widths are constant. N increases with time because no chromatographic system has an ideal behavior; and even with gradient elution the peak widths increase gradually. Therefore the Number of Possible Peaks, NPP, is a more realistic value than the peak capacity, n:

$$NPP = \Delta\, t_R / W_n\text{-}W_1\,.\,\mathrm{Ln}(W_n/W_1)$$

with the retention time interval $\Delta\, t_R$ covering peaks 1 to n (including their widths) and peak widths w.

The figure presents a first peak at 2 min, or 120 s, of width 6.2 s (N = 6000) and a last at 3.33 min, or 200 s, of width 8.4 s (N = 9000). Thus the retention time interval runs from (120-3.1) s to (200+4.2) sor a span of 87.3 s. With these data NPP = 12 as shown in the computer simulation with peaks of resolution 1.0 [165]



$$NPP = \frac{\Delta t_R}{w_n - w_1} \ln \frac{w_n}{w_1}$$

Figure-5

4.7 Statistical Resolution Probability: Simulation

Even with mixtures of moderate complexity it is not realistic to expect that all compounds can be separated without an extra effort at optimization. The opposite is the general rule - we are forced to assume that there are more compounds than visible peaks. If the separation problem does not deal with homo logs or with molecules of another type of regularity, the retention times (or k values) show a random distribution, therefore the chance of peak overlap is high. This statistical resolution probability, P, for a single compound of the mixture is defined by

$P \approx e^{-2m/n}$

where m = true number of compounds in the mixture and n = peak capacity or, for real chromatograms, NPP = Number of Possible Peaks .

Example: breakthrough time *to* = 40 s, maximum retention time = 363 s, thus k_{max} = 8.08 and theoretical plate number N = 5000, giving a peak capacity of n = 40. The sample mixture consists of 10 compounds, i.e m = 10. The probability that a certain compound will be separated from its nearest neighbor with a resolution $R \geq 1$ is:

$$P \approx e^{-2\ 10/40} = 0.61 = 61\%$$

This means that we expect that only 6 out of the 10 compounds will be totally resolved! Because this prognosis is based on statistics, it is quite possible that in a given case more or fewer than 6 compounds will have a resolution greater than 1 on both sides.

Computer simulation with random numbers:

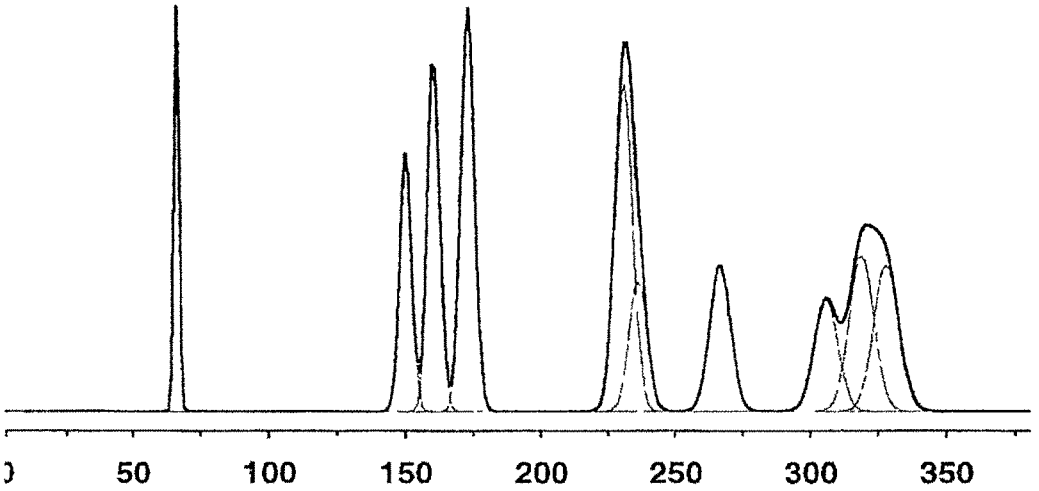
Retention time window between 40 and 363 s

Theoretical plate number N = 5000 (constant, less realistic)

Peak capacity n = 40

Number of compounds m = 10

Number of visible peaks = 8, including, however, 1 doublet and 1 triplet Number of peaks with resolution R < 1 = 5 [165]



$$P \approx e^{-2\,m/NPP}$$

Figure-6

4.8 Statistical Resolution Probability: Example

A mixture of six compounds without chemical similarity needs to be separated. With hexane/tetrahydrofuran (top) and with hexane/ dichloromethane (bottom) only five peaks are visible, only hexane/ tert-butyl methyl ether separates them all (middle). If no extra optimization is tried, in two of the three cases fewer peaks are found than are really present.

If the chromatogram is recorded by a computer, the dataJrom all the peaks are easily obtained. In the examples shown the theoretical plate number increases from ca. 8000 to ca. 13000 between the first and last peak. The Number of Possible Peaks NPP is, from top to bottom, 19, 27, and 47 (NPP is larger the longer the maximum retention time); the calculated statistical resolution probabilities are 0.54, 0.64, and 0.78. The real resolution probabilities are, from top to bottom, 0.66 (4 of 6 peaks are totally resolved), 1.0 (all peaks are resolved), and 0.33 (only 2 of 6 peaks are resolved;the resolution is 0.9 for peaks 5 and 4).

The example shows that one possible means of tracking down hidden peaks is to run the sample with several different phase systems. Another method is to use the peak purity function of the diode array detector; this only fails if the unresolved compounds have identical UV spectra or exactly identical retention times.

Chromatographic conditions:

Sample:1,4-diphenylbutane, 2-phenylethylbromide, phenetole (phenyl ethyl ether), trans-chlorostilbene oxide, nitrobenzene and 4-chlorobenzophenone dissolved in hexane and a little THF

Column : 3 2mm x 25cm

Stationary phase : LiChrosorb SI 60, 5 /lm (silica)

Mobile phase : as indicated, 1 mL min^{-1}.

Detector: UV 254 nm [165]

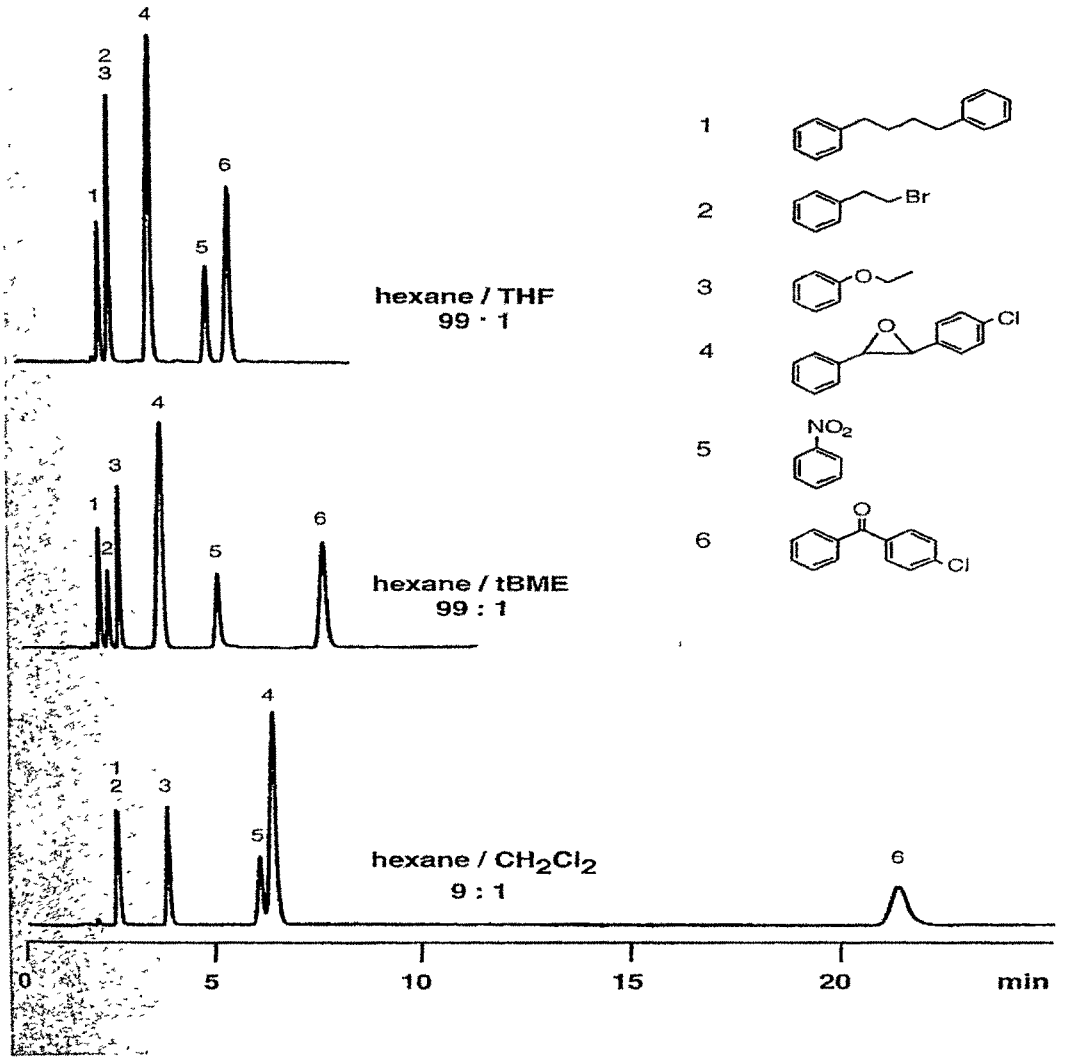


Figure-7

4.9 Precision and Accuracy of an Analytical Result

It is important to distinguish between precision and accuracy whenever an analytical result is evaluated. During method development both aspects must be optimized and it is not acceptable to neglect one of them.

High precision means that the scatter of data is low if the analysis is repeated several times. It is easily calculated as the standard deviation or coefficient of variance of the obtained data It describes the random deviations of the method

High accuracy means that the analytical result deviates only slightly from the true value. It is a measure of the systematic deviations of the method. If the true value is not known, which is usually the case, no statement about accuracy can be made. Knowledge about the accuracy of one's work can be obtained by analysis of reference materials, inter-laboratory tests, and analysis of a particular sample by another method . Unfortunately, accuracy tends to decrease with decreasing concentration of the analyte in the sample .

Accuracy is more important than precision A method with poor precision but good accuracy, as symbolized by the bottom left diagram, needs to be repeated many times but the mean will be accurate. In contrast with such a case, methods with high precision but lacking accuracy, as at the top right, are extremely unfavorable, there is a real danger that the analyst will be happy with the result, owing to its low standard deviation, and stop looking for sources of error.
In any case the goal is precise and accurate analyses, as at the bottom right, with greater attention to accuracy. For this it is, however, necessary to make use of one's imagination and to take a self-critical approach to one's work [165]

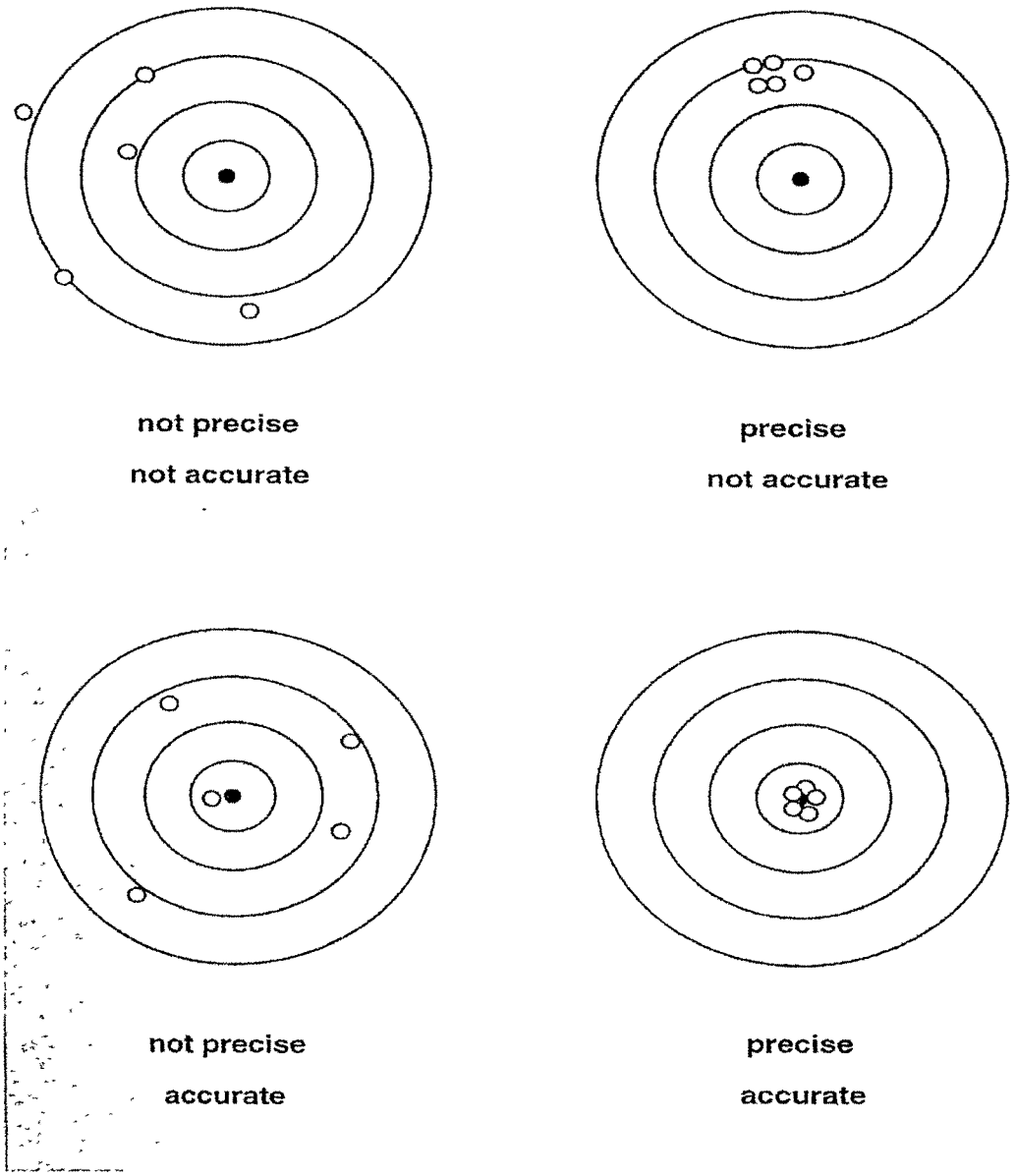


Figure-8

4.10 Standard Deviation

The standard deviation of an analytical result may only be calculated if random deviations occur. The data must be scattered without showing a trend, and systematic effects (due to poor sample preparation, wrong method, a non-adjusted instrument, or

even the analyst) must be absent. The true standard deviation σ is only obtainable from an unlimited number of data; what is calculated from a limited set is the is the "estimate of the standard deviation" *s*[1]. Nevertheless, s is usually called the standard deviation. s divided by the mean and multiplied by 100 gives the relative standard deviation in percent, also called the coefficiant of variation CV. The use of a calculator enables it to be determined h with ease.

The significance of the standard deviation is as follows: 68 % of the data are within ± s (only 68%!), 95% are within ± *2s*, and 99.7% are within ± *3s*. This means that almost one third of the data (32 %) deviate from the mean by more than the amount indicated by the value of s.

Example of a data set: 19.92, 19.78, 19.17, 19.03, 19.33, 19.87, 19.83, 19.59, 18.97, 19.31, 19.37, 19.19, 19.25, 19.67, 19.46, 19.27, 19.21,19.97, 19 76, 19.57. This gives a mean of 19.48 with a standard deviation of ± 0.31 or ± 1.6%.

Meaning: 68% of the data can be expected between 19.17 and 19.79

32 % of the data are presumably smaller than 19.17 or larger than 19.79

95 % of the data can be expected between 18.86 and 20.10

5 % of the data are presumably smaller than 18 86 or larger than 20.10

$$s = \pm \sqrt{(d^2_1+d^2_2+d^2_3+.....+d^2_n)/n-1}$$

d: deviation of a data point from the mean

n: number of data [165]

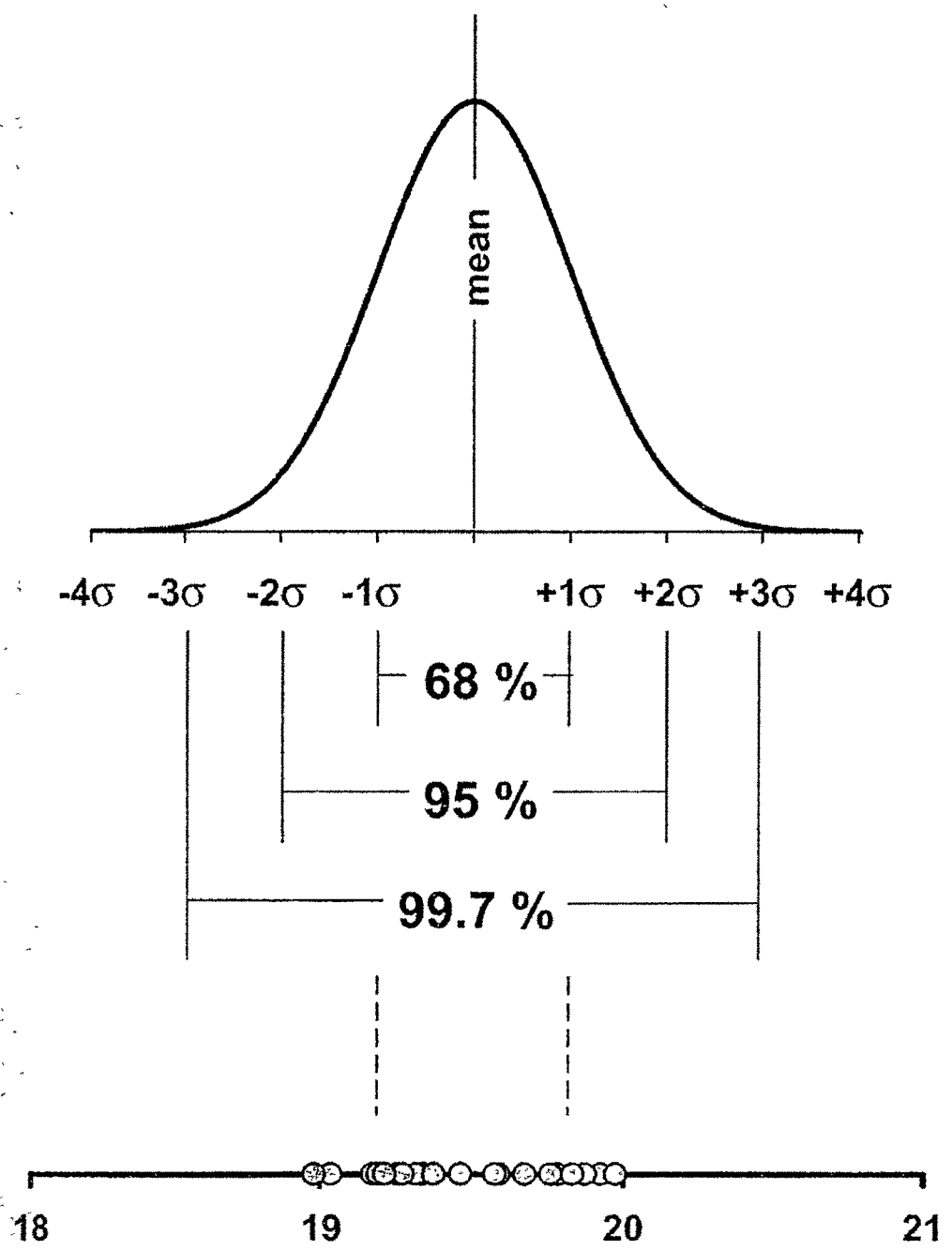
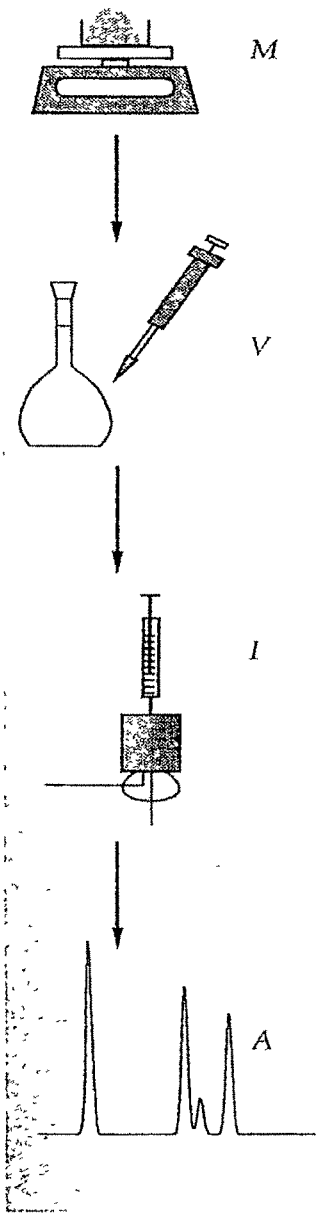


Figure-9



$$\frac{u(R)}{R} = \sqrt{\left(\frac{u(M)}{M}\right)^2}$$

$$\frac{u(R)}{R} = \sqrt{\left(\frac{u(M)}{M}\right)^2 + \left(\frac{u(V)}{V}\right)^2}$$

$$\frac{u(R)}{R} = \sqrt{\left(\frac{u(M)}{M}\right)^2 + \left(\frac{u(V)}{V}\right)^2 + \left(\frac{u(I)}{I}\right)^2}$$

$$\frac{u(R)}{R} = \sqrt{\left(\frac{u(M)}{M}\right)^2 + \left(\frac{u(V)}{V}\right)^2 + \left(\frac{u(I)}{I}\right)^2 + \left(\frac{u(A)}{A}\right)^2}$$

Figure-10

4.12 Calibration Curves

Quantitative analysis demands calibration curves which are linear, run through the origin, and have low scattering of the data points . Unfortunately obtaining such a perfect calibration graph is not a matter of course because every method is prone to a number of errors. Different and multiple deviations from the ideal are possible:

Not enough data points: two points only do not result in a calibration curve! The minimum is three points, and five points should be the rule. (This type of error is called sloth and not pitfall.)

High scattering: a repeated determination of the calibration curve from the very beginning will make it clear whether the deviations are of a random or systematic nature. Random effects give other data points, systematic ones will reproduce them at more or less the same spot. In any case the fluctuations should be minimized

Non-linear curve: if the non-linearity cannot be traced to random effects and if it persists after a thorough checking of the method , one has no other choice than to use this less favorable curve although attempts should always be made to improve it.

Inadequate spacing of the data points: the calibration points must be spaced evenly over the x axis range. Otherwise the uncertainty is too high in the range without points.

Incomplete calibration curve: this curve does not cover the full span of content which might occur in the samples. It is never permissible to extrapolate a calibration curve to a range which was not investigated

Calibration curve with proportional-systematic deviation: the slope of the curve is too high (as shown) or too low. This error ~ not easy to recognize but it affects accuracy! The reason for the deviation can be trivial, e.g a dilution error, or very unexpected and difficult to explain.

Calibration curve with constant-systematic deviation: this curve does not run through the origin but is too high (as shown) or too low The same recommendations are valid as given above for the non-linear curve [165]

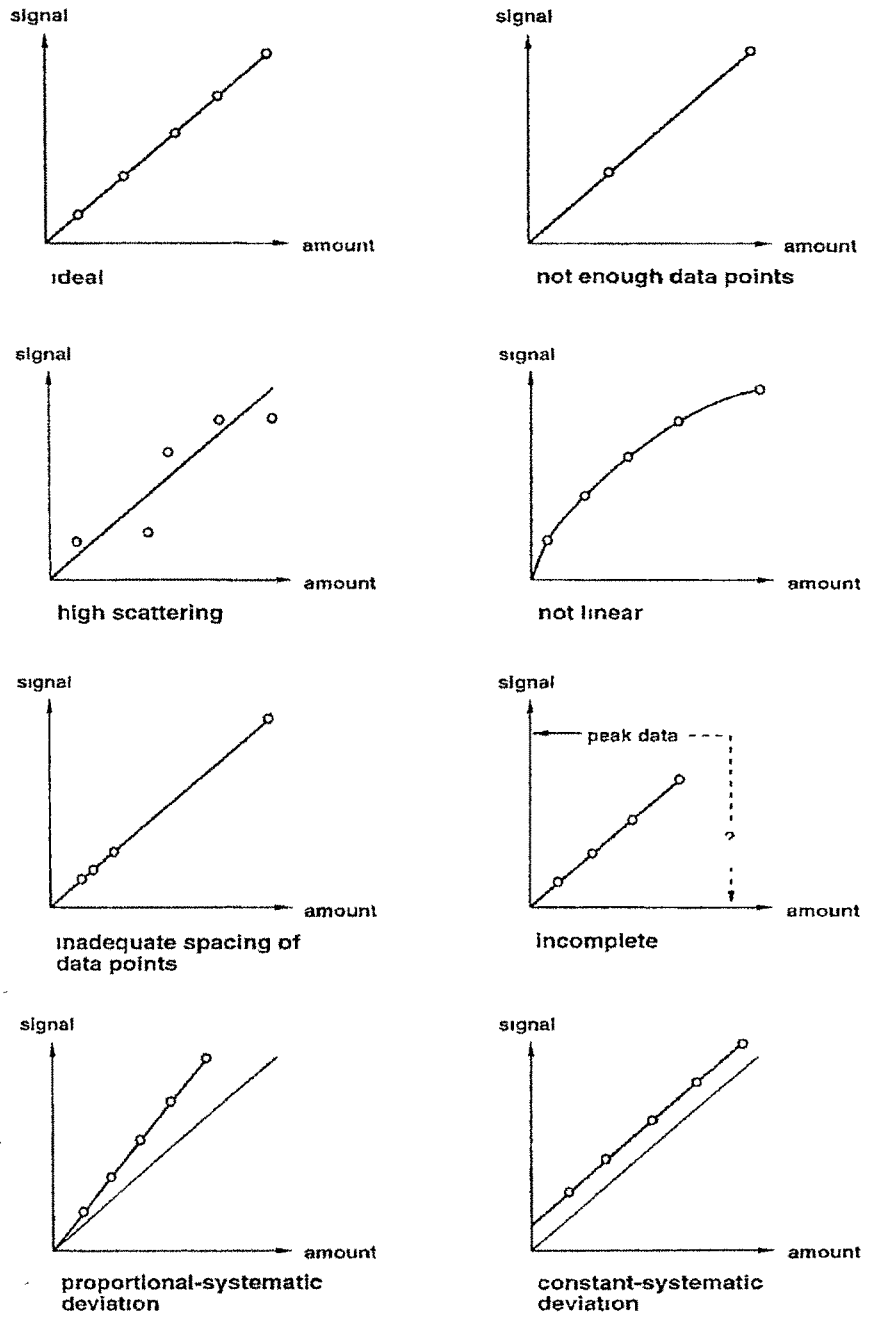

signal
amount
ideal
signal
amount
not enough data points
signal
amount
high scattering
signal
amount
not linear
signal
amount
inadequate spacing of
data points
signal
peak data
amount
incomplete
signal
amount
proportional-systematic
deviation
signal
amount
constant-systematic
deviation

Figure-11

4.13 The HPLC Instrument

The finer the particles of the stationary phase the higher is the number of theoretical plates per unit length and the higher is the optimum flow rate of the mobile phase Therefore HPLC (high performance liquid chromatography) uses stationary phases of 10 μ*m* diameter or less. However, such fine column packings result in high flow resistance, which makes it indispensable to use a pump to transport eluent through the column. A detector enabling observation of the eluted peaks is essential Some basic requirements of the individual parts of the HPLC instrument are as follows.

Pump: pulse-free transport of the mobile phase with digital adjustment of the flow rate. The flow must be independent of the back pressure.

Injector: accurate and precise injection of the desired volume must be possible The carry-over between consecutive injections must be negligibly small.

Detector: low noise wide linear range . Neither cell volume nor time constant must contribute markedly to band broadening. The detector can be highly specific (e.g. the electrochemical detector at a defined potential) but also totally non-specific (e.g. the refractive index or the light-scattering detector) .

Connecting capillaries: because it is necessary to prevent band broadening, the capillaries from injector to column and from column to detector should be very thin (maximum inner diameter 0.25 mm) and short.

Data processing unit: data systems are very convenient and can be used in a quality-assured environment if they are adequately designed. They do not, on the other hand, increase the quality of the analytical results above that which can be obtained from a much simpler integrator.

In addition to the items shown here, the HPLC instrument can also be equipped with a gradient system, an auto sampler, and a column thermostat. [165]

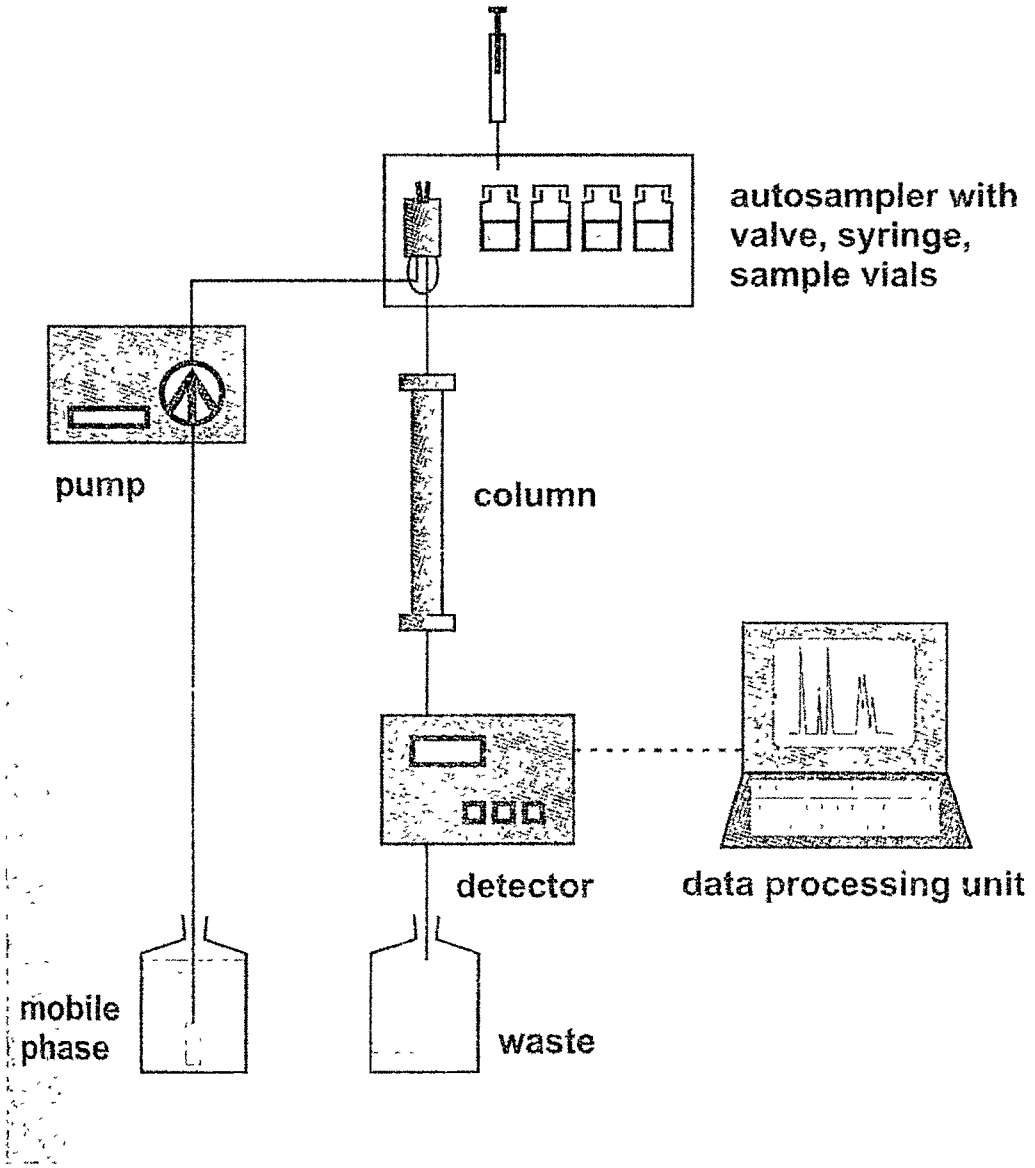

autosampler with
valve, syringe,
sample vials
pump
column
detector
data processing unit
mobile
phase
waste

Figure-12

4.14 The Detector Response Curve

As users of a detector, we expect the instrument to yield a signal which is proportional to the amount of sample injected over a wide range of mass or concentration. This so-called linear range is represented by a straight line in a plot of signal (e.g. in mill volts) as a function of mass or concentration. It should stretch over as wide an interval of mass or concentration as possible Under a certain threshold value the heights of the peaks are very small compared with the noise level, making it impossible to perform an analysis. This is the detection limit, which should be as low as possible but which is directly related to the noise intensity. The detection limit is not identical for qualitative and quantitative analysis. The figure shows a peak approximately at the limit of unambiguous qualitative detection.

Within the linear range the peaks are recorded with their true shape. If more sample is injected than is allowed by the linear range the signal is too flat and not in accordance to its true elution profile. In fact, although there is still a dependence of signal on mass or concentration this relationship is weak and the peaks are not high enough. Accurate quantitative analysis is not possible If the sample amount is even larger, then the dynamic range also is exceeded and the signal is saturated With a UV detector the photodiode behind the cell does not register any light; with an electrochemical detector the maximum current is running. Under such circumstances the tops of the peaks are cut off horizontally.

A calibration curve must be confined within the linear range . In principle it would be possible to expand it to the upper dynamic range but this gives uncertain or even inaccurate results. If necessary the sample needs to be diluted to avoid this. It is totally wrong to extrapolate a calibration curve, starting from a number of data points within the linear range, the upper end of the linear range must be known in order to be sure that samples of high concentration can also be analyzed accurately

The x axis is for 'mass or concentration' because detectors can be mass or concentration sensitive UV detectors measure concentration (they are concentration sensitive); if the flow of the mobile phase is stopped at the moment when a peak is passing through the cell the signal remains unchanged In contrast with such devices

coulometric detectors measure a mass flow (they are mass sensitive); if the pump is stopped the signal disappears within a short time. [165]

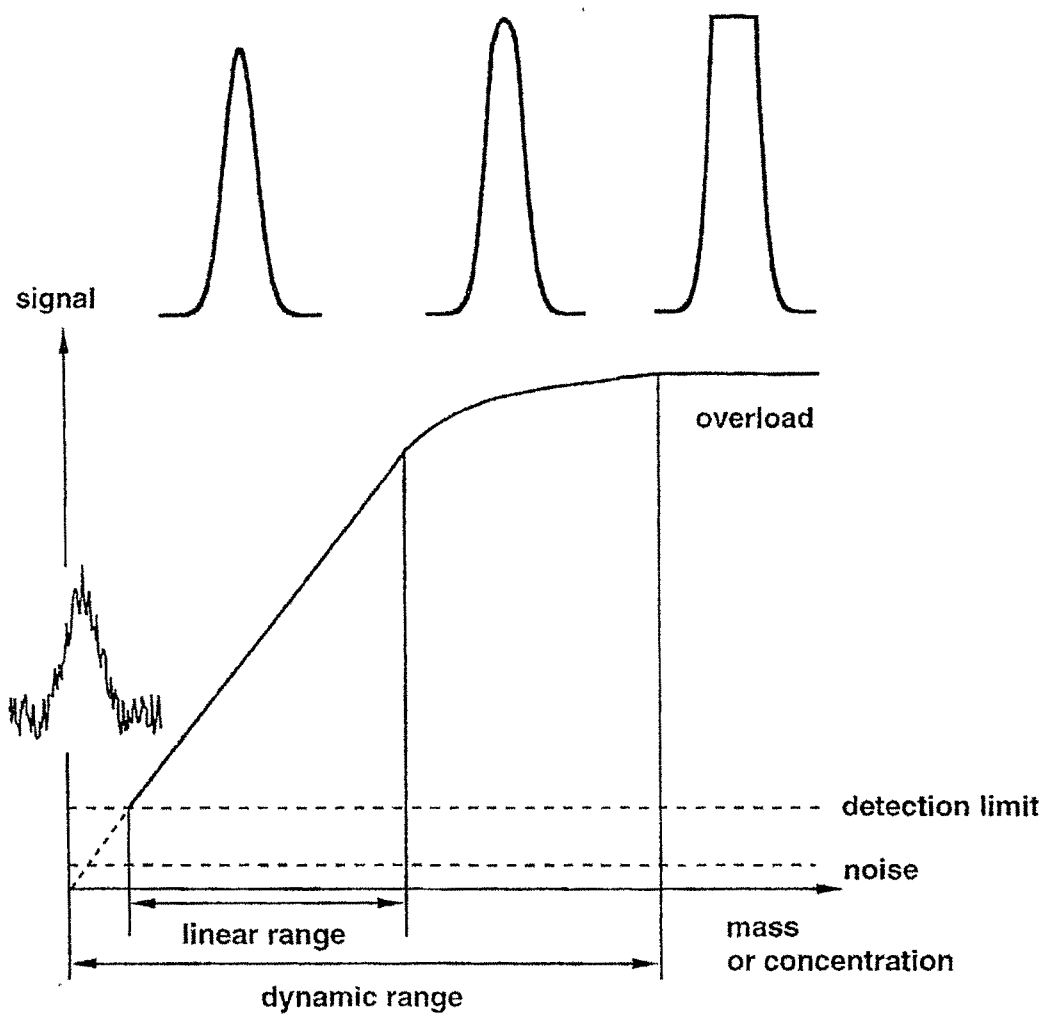


Figure-13

4.15 Noise

Short-term fluctuations of the baseline are called noise. Noise affects quantitative analysis and the detection limit in trace analysis. Therefore it is necessary to make every effort to suppress noise: mobile phase degassing (this is especially important with polar eluents and with systems which mix the solvents within the HPLC instrument), the use of a well-maintained, pulse-free pump or of extra pulse-dampeners, the build-up of a small additional pressure drop at the detector output (e.g. by connecting a long capillary of 0.25 mm inner diameter), shielding from interfering electrical fields, and protection of the instrument from draught. Noise can also be suppressed by increasing the time constant of the detector and integrating system but this method impairs the detection of narrow peaks and can only be recommended if both the noise frequency and the width of the narrowest peaks are known. Yet even under the best conditions noise is present which comes from the electronics and which cannot be lower than some threshold level.

The detector response curve disappears at its lower end within the noise and this point determines the detection limit for quantitative and qualitative analysis. The figure presents both limits as they are often defined in the literature: For precise quantitative analysis the signal-to-noise ratio (S/N) should not be lower than 10, as shown by the large peak. This is the limit of quantitation (LOQ). Depending on the analytical demands this value may be too low and it may be better to define the LOQ at S/N = 50 For unambiguous qualitative analysis S/N must not be lower than 3, as for the small peak; this is the limit of detection (LOD). This value also may be too low because in practice the noise is less regular than that shown in the figure, which was calculated by use of a computer generating random numbers with. in a given span. [165]

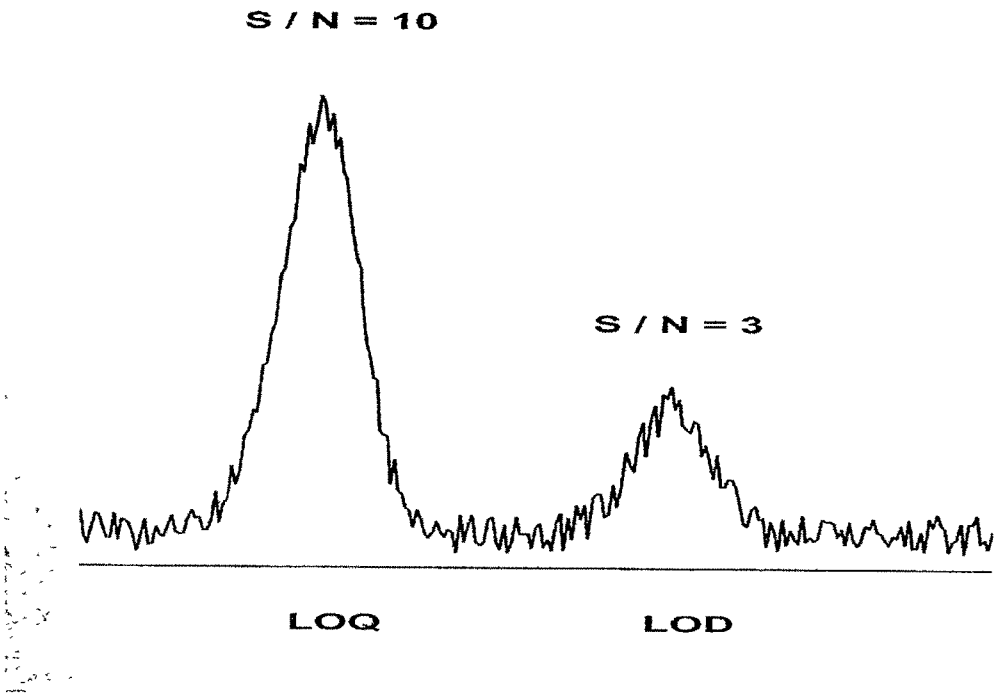


Figure-14

4.16 Causes and Effects Presented as an Ishikawa Diagram

This diagram is about things that can go wrong when performing HPLC analyses. Every operation or part of the set-up, from the solvent to the presentation on the screen, can be the cause of an erroneous result. The hierarchy of causes which lead to a certain effect can be represented as an Ishikawa diagram (cause-and-effect diagram).

This shows the factors which influence the shape, size, and position (in terms of its retention factor) of a single chromatographic peak. They can be grouped into the effects stemming from injection, separation, detection, and integration. The resulting diagram may be confusing at first sight but there is no question that it could be drawn in even more detail. The complexity of the presentation makes clear that only excellent professional knowledge leads to excellent analyses.

The principle of a quantitative chromatographic analysis consists in the comparison of reference and sample peaks. This approach leads to a certain simplification of the problem as presented in the drawing. If identical volumes of sample and reference

solutions are injected, the calibration of the auto sampler is irrelevant. Similarly, detection accuracy can now be of minor importance; if two peaks are, e.g., detected at the wrong wavelength, they will both deviate from their "true" values by the same relative amount. If both peaks are of similar size, their shapes and signal-to-noise ratio will be similar, and the integration parameters will be the same anyway. What is important under these circumstances is the repeatability of all steps, including the prerequisite that the mobile phase must not change its properties from one injection to another [165]

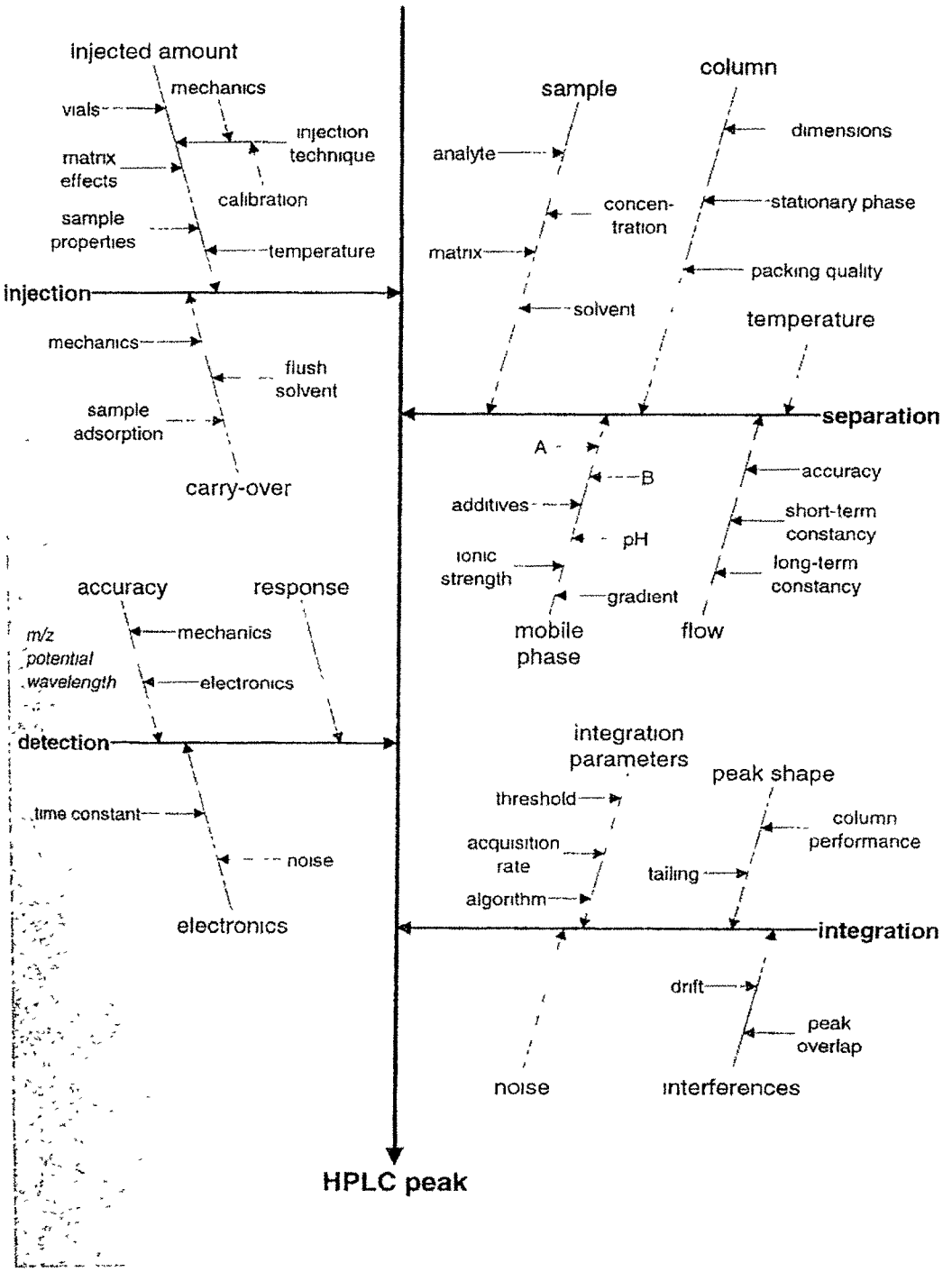

injected amount
mechanics
vials
injection technique
matrix effects
calibration
sample properties
temperature
injection
mechanics
flush solvent
sample adsorption
carry-over
column
sample
dimensions
analyte
concen-tration
stationary phase
matrix
packing quality
solvent
temperature
separation
A
B
accuracy
additives
short-term constancy
pH
ionic strength
long-term constancy
gradient
mobile phase
flow
accuracy
response
m/z
potential
wavelength
mechanics
electronics
detection
integration parameters
peak shape
threshold
column performance
acquisition rate
tailing
algorithm
integration
drift
peak overlap
time constant
noise
electronics
noise
interferences
HPLC peak

Figure-15

4.17 Column Tests

After purchase each HPLC column should be tested. According to internal guidelines the test is repeated at regular time intervals and whenever column deterioration is suspected. The test results are kept (either physically or as computer files) for as long as the column is in use.

The test can be performed with standard compounds (under isocratic conditions) or with a typical sample for which the column will be used. With the first type of test it is advisable to calculate some figures of merit whereas the second type is primarily used to judge the quality of the separation with regard to the resolution of a critical peak pair or to the peak shape.

The upper chromatogram shows a test with standard compounds. The calculated figures of merit are. theoretical plate number N of the last peak = 14500, reduced plate height h of the last peak = 3.0, tailing T of the last peak = 1.3, and reduced flow resistance $\varphi = 970$.

The lower chromatogram shows the separation of PTH amino acids on the same column. It is known from experience that the separation of 19 amino acids as found here is only possible on an excellent column.

Chromatographic conditions:

Column: 2.1 mm x 22 cm

Stationary phase: PTH *C-18,* 5µm (reversed phase C18)

Top: Sample: benzyl alcohol, benzaldehyde, and benzoic acid methyl ester

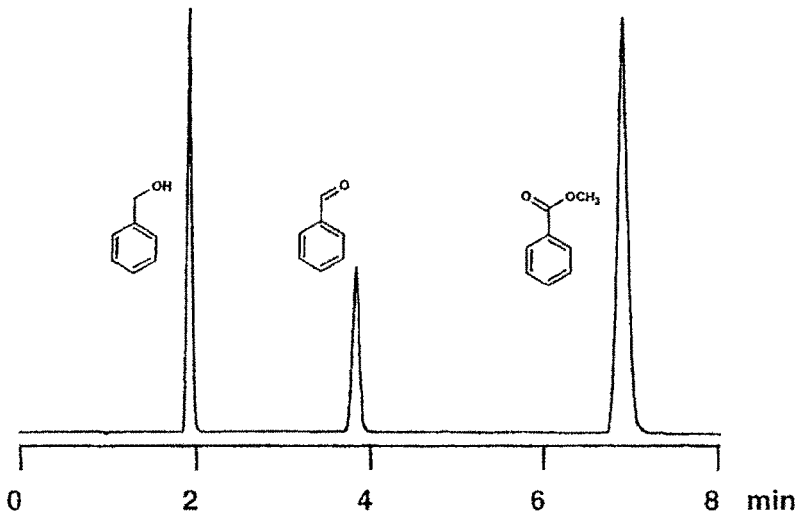
Mobile phase: water/acetonitrile 6 : 4, 0.4 mL min^{-1}

Pressure . 184 bar

Bottom: Sample: phenylthiohydantoin amino acids

Mobile phase: gradient with water, tetrahydrofuran, acetonitrile, isopropanol and additives, 0.21 mL min^{-1}

Detector:UV 269 nm [165]


OH
O
O
OCH3
0
2
4
6
8
min

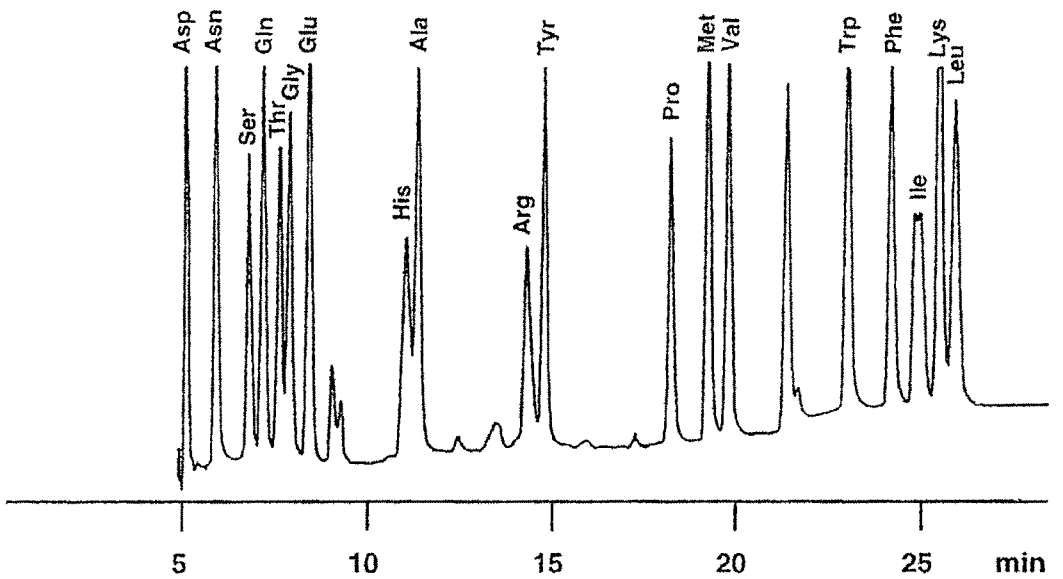

Asp
Asn
Ser
Gln
Thr
Gly
Glu
His
Ala
Arg
Tyr
Pro
Met
Val
Trp
Phe
Ile
Lys
Leu
5
10
15
20
25
min

Figure-16

4.18 Apparatus Tests

Accurate, precise, and reproducible analytical results can only be obtained if the various functions of the HPLC system are tested and adjusted regularly. There are no generally mandatory procedures for such tests because every laboratory or company is free to prescribe how often and to what extent they shall be performed. The results need to be noted in a log-book. Binding rules should, moreover, state how to proceed if the specifications are not reached. It is possible to hire a technician of a specialized company for the tests.

A comprehensive test includes the following functions:

- For the pump, the flow accuracy (e.g., better than 5%), the short term and the long-term flow constancy (e.g., better than 0.5% and 0.2 %, respectively).

- For the injector, the reproducibility (e.g., better than 0.5%); it is necessary to distinguish between partial and total filling of the loop .For the auto sampler, the accuracy and the injection carry-over also.

- For the detector, the noise (e.g., less than 0.04mAU, 1 AV = 1 absorbance unit) and the wavelength accuracy (e.g., better than 2 nm) of UV or fluorescence detectors.

- For the gradient system, the accuracy and reproducibility of the profile. [165]

Apparatus Tests

Pump: flow accuracy, short-term flow constancy, long-term flow constancy

Injector: reproducibility

Autosampler:reproducibility , accuracy, carry-over

Detector:noise,wavelength accuracy

Gradient system: accuracy, reproducibility of the profile

Figure-17

4.19 Wavelength Accuracy of the UV Detector

In a laboratory which works in accordance with a quality assurance system it is necessary *to* check the wavelength adjustment of the DV detector regularly .Such a test is also recommended for all other laboratories. Various test compounds can be used but one with distinct and narrow absorbance maxima is always *to* be preferred over another with a single broad maximum, as is often found in organic molecules. Benzene (as hexane solution or even better as vapor) could be used for calibration in the DV but because of health considerations this cannot be recommended.

An interesting compound is erbium per chlorate which has several sharp maxima in aqueous solution They include the important wavelength of 254.6 nm and several bands in the visible region.

Terbium perchlorate with an absorption maximum at 218.5 nrn can be used for calibration at low wavelengths. [165]

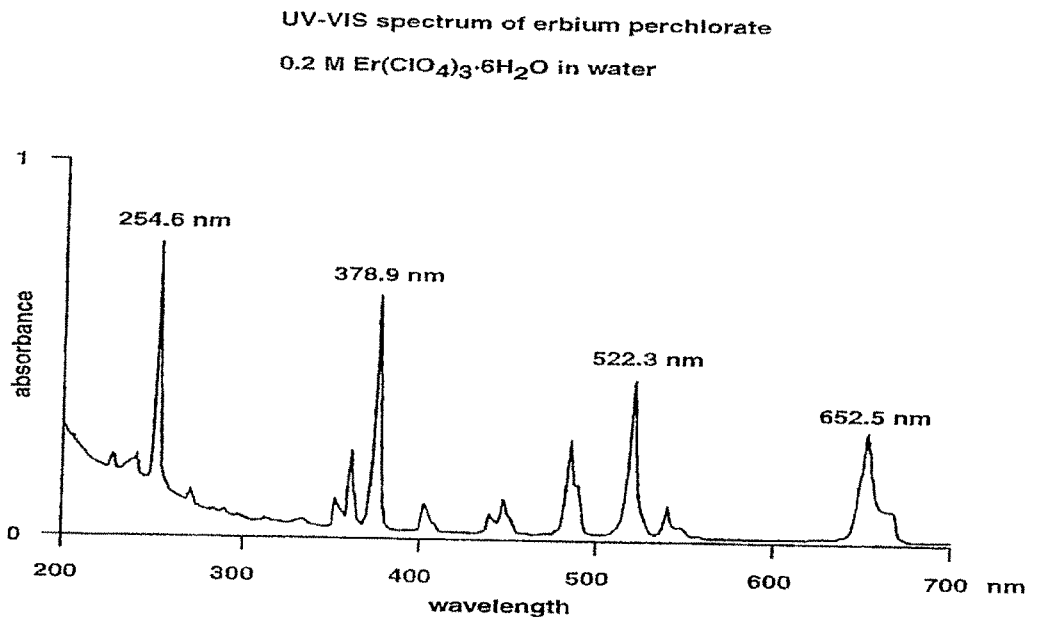


Figure-18

4.20 Rules for Passing On an HPLC Method

It is necessary to note in detail very many procedures and data if one wants to use a method in another laboratory. This applies to internal methods within a company as well as to scientific publications.A problem on its own is always the sampling procedure. It is also possible that sample storage and preparation will influence the analytical result. These need detailed descriptions, including the preparation of the injection solution as the last step.

The preparation of the mobile phase must be described in detail: solvent mixing ,quality and amount of reagents ,pH .The temperature of the column should also be stated (otherwise add the remark 'separation at ambient temperature, the influence of temperature was not investigated').Extra-column volumes and, for gradient separations, the dwell volume are part of a method description Column dimensions and the precise name of the stationary phase, in extreme cases even the batch need to be given as a matter of course as well as the volume flow rate and, if applicable, the gradient run time .

If a UV or fluorescence detector is used the wavelength must be known and detector calibration at regular time intervals is a prerequisite. The integrator parameters can influence data acquisition. With data systems which allow re-integration of the chromatogram this is less important. Unfortunately untoward surprises are almost to be expected if a method comes from academia because university institutes rarely in stall a formal quality assurance .

Specifications which need to be known for successful method transfer
Sampling, sample storage, sample preparation, Sample solvent
Mobile phase preparation, reagent quality
Temperature
Extra-column volumes
Dwell volume of gradient separations
Column dimensions
Volume flow rate of the mobile phase
Detailed identity of the stationary phase
Detector data, Integration parameters

Figure-19

4.21 Method Validation

Method validation is the process which proves that an analytical procedure is suited for the intended purpose. Only with validated methods is there a guarantee that the laboratory produces accurate and precise analytical results. Lucky hits and trimmed data are not reconcilable with validation.

A prerequisite for validation is instruments which have passed the apparatus test. All software which is used needs to be designed such that results cannot be altered and no files can disappear. The laboratory staff works according to the rules of quality assurance. Only Standard Operating Procedures (SOPs) are used as methods of analysis .

Validation is the knowledge and documentation of the selectivity (perhaps specificity), range, linearity, quantitation limit, detection limit, precision, accuracy, and ruggedness of a certain procedure The corresponding definitions can be found on the opposite page.

Depending on the analytical problem it is perhaps not necessary to determine all these parameters (although this is the rule) If, e.g., the main product which constitutes 10 to 90% of a formulation is to be analyzed, one is not interested in the quantitation limit.

Control charts can be extremely helpful during routine use of a method. If the chart shows that the analytical results are free from outliers for a long period of time it might be possible that multipoint calibration is not necessary because linearity is not a problem. Under such conditions the laboratory can switch to one-point calibration but the personnel will check the results by the continuous use of the control chart.

Validated Procedures

Validation means knowledge and documentation of

Selectivity: The compound of interest can also be identified and quantified in the presence of other (similar) compounds

Specificity: Only the compound of interest is determined whereas other components or features of the sample do not influence the result.

Range: Range of concentration or mass for which the procedure can be used with appropriate precision and accuracy.

Linearity: Straight calibration curve, Le. the data points are directly proportional to concentration or mass.

Quantitation limit: Lowest concentration or mass which can be quantitated precisely and accurately (often 10 x noise level).

Detection limit: Lowest concentration or mass which can be detected qualitatively (often 3x noise level).

Precision: Standard deviation. Differentiate between repeatability (same column, same instrument, same laboratory...) and reproducibility (not same laboratory...).

Accuracy: Difference between found and true value.

Ruggedness: Insensitivity to small fluctuations of the parameters of the procedure

4.22 Some Elements of Validation

Some elements of validation which are discussed in 4.21 can be drawn into the presentation of a calibration curve for the purpose of visualization.

Limits of quantitation and detection: Here the detection limit with approx. threefold noise level is drawn. A peak at the quantitation limit should have a height of approx. ten times the noise level .

"True value" (black point): The true value of an analysis, i.e. the true concentration of analyte, is unknown by definition. Besides the fact that it would be necessary to perform a large number of measurements in order to get a reliable mean the analyst can never be sure about the absence of unknown and uncontrollable systematic effects which could affect the result.

Trueness: The difference between the found and the true value Since the true value is not known, the trueness is unknown as well.

Repeatability (grey points): In the situation shown here the repeatability is the standard deviation of several peak sizes (area or height) which were obtained by the multiple injection of a certain sample solution.

Linearity (white points)· The distribution of the data found with the calibration runs shows that it is justified to postulate a linear relationship between the concentration of the analyte and the peak size. The points are dispersed in a random manner above or below the regression line .

Specificity: The detail from the chromatogram shows that the analyte peak seems to be free from interfering peaks. During the validation it is necessary to investigate by diode array detection, mass spectroscopy and/ or another analytical method if a co elution can be excluded with high probability.
[165]

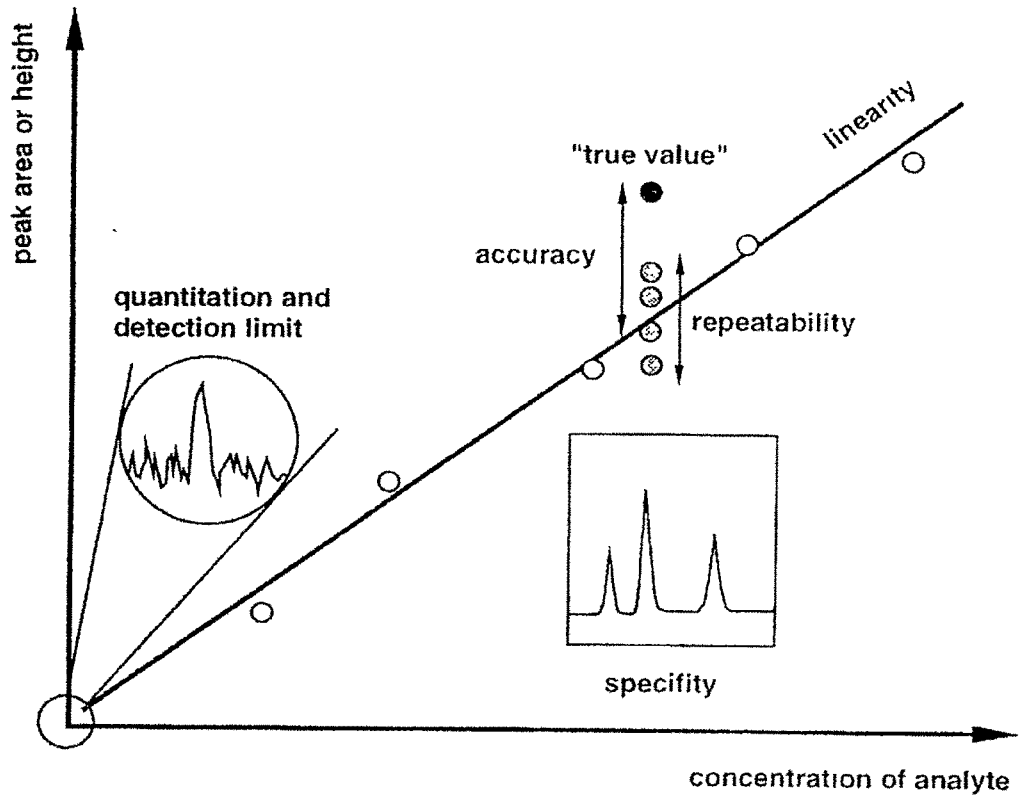


Figure-20

4.23 Measurement Uncertainty

The document "Quantifying Uncertainty in Analytical Measurement" gives a useful (and in some cases mandatory) guideline on how to determine the measurement uncertainty of a quantitative chemical analysis. The schematic procedure is presented in the graph.

Specify measurand: The standard operating procedure must be understood. (If not, write it in a clearer and more comprehensible style or educate the personnel.) The complete equation of the measurand must be known. In the case of a one-point calibration HPLC analysis without sample preparation it is of the type·

$$C = A_s/A_r.m_r.P_r/V_r \,.\, V_s/m_s$$

with c: concentration of the analyte in the sample (measurand); *A:* peak area; m: mass; P. purity; *V·* volume of measuring flask; s refers to the sample and r to the reference

Identify the uncertainty sources An Ishikawa diagram can be very helpful .It is strictly based on the equation of the measurand.

Quantify the uncertainty sources: Look for experimental data (such as repeatabilities, validation data) and/or published data (such as purities, technical data of the glassware and pipettes). Transfer the data into standard uncertainties.

Calculate the combined standard uncertainty *Uc*: For the equation given above it is calculated as follows (as relative value)·

$$Uc(c)/c=\sqrt{\{u(A_s)/A_s\}^2}+\sqrt{\{u(A_r)/A_r\}^2}+\sqrt{\{u(m_r)/m_r\}^2}+$$
$$\sqrt{\{u(P_r)/P_r\}^2}+\sqrt{\{u(V_r)/V_r\}^2}+\sqrt{\{u(V_s)/V_s\}^2}+\sqrt{\{u(m_s)/m_s\}^2} \quad [\,165\,]$$

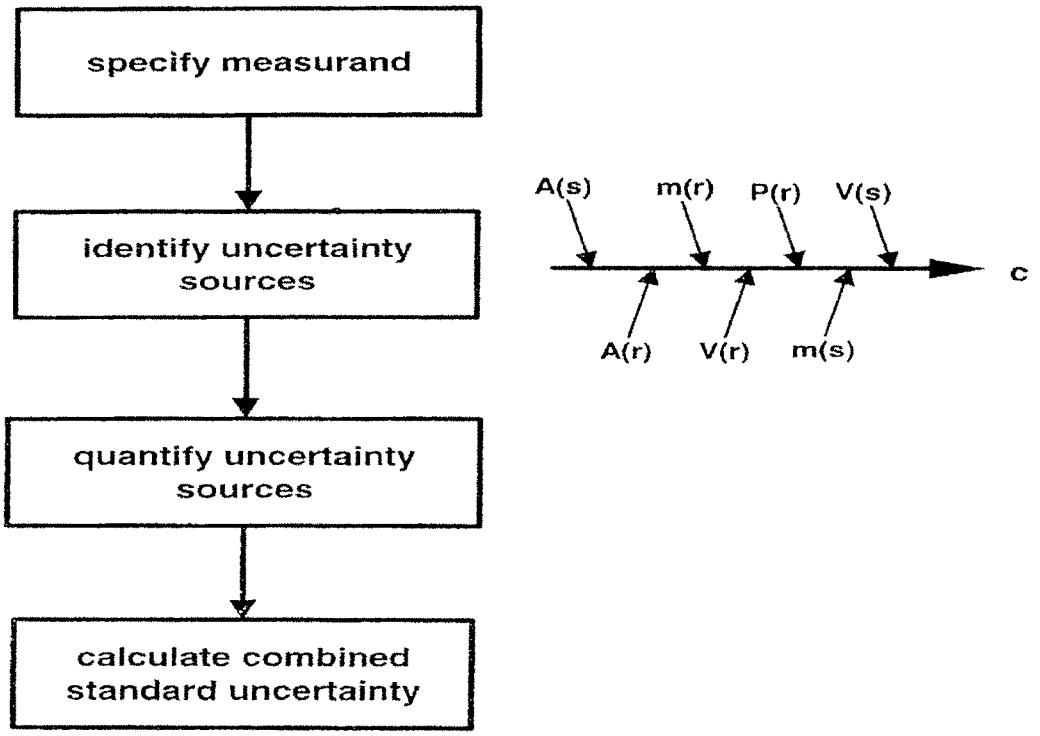

specify measurand
identify uncertainty sources
quantify uncertainty sources
calculate combined standard uncertainty
A(s)
m(r)
P(r)
V(s)
c
A(r)
V(r)
m(s)

Figure-21